Kinetic and Structural Studies on the Interaction of Proteinase Inhibitor from *Dolichos biflorus* (Horse Gram)

P. R. Ramasarma,[†] A. G. Appu Rao,[‡] and D. Rajagopal Rao[*,†]

Department of Biochemistry and Nutrition and Department of Protein Technology,
Central Food Technological Research Institute, Mysore 570 013, India

One of the major components of multiple inhibitors from *Dolichos biflorus* (horse gram) (HGPI) inhibits both bovine trypsin and chymotrypsin, resembling other Bowman–Birk-type protease inhibitors. Ultraviolet absorbance measurements indicate the presence of two tyrosine residues, the absence of tryptophan, and the dominance of disulfide linkages in the molecule. The intrinsic fluorescence emission maximum (λ_{max} 336 nm) is due to the tyrosine residues in the hydrophilic environment. Disulfide linkages and tyrosine residues contribute to the near-ultraviolet circular dichroism spectra. Far-ultraviolet circular dichroism measurements indicate the absence of any helical structure, 31% β structure, and the rest aperiodic structure. Aromatic amino acids are involved in the interaction of the inhibitor with trypsin or chymotrypsin. The equilibrium constants for the interaction of HGPI with chymotrypsin/trypsin were 2.9×10^5 and 5.2×10^5 M^{-1}, respectively, with an estimated stoichiometry of 1:1 with either of the enzymes.

INTRODUCTION

Proteinase inhibitors, comprising one of the abundant classes of proteins found throughout all life forms, have been identified for all classes of proteases (serine, cysteine, metallo, and aspartyl). The inhibitors of serine proteinases from plant sources have been isolated, characterized, and recently implicated to play a predominant role in natural plant defense and infection processes. These proteinase inhibitors have been studied extensively at the structural and mechanistic level and at the level of gene regulation (Ryan, 1989, 1990), and it has been recently recognized that the plant proteinase inhibitors are known to have strong anticarcinogenic activity in *in vivo* and *in vitro* cancer model systems and could serve as chemopreventive agents in the treatment of cancer (Birk, 1976; Troll and Kennedy, 1989). Proteinase inhibitors have also been used in studies relating to understanding Alzhemier's disease and autoimmune diseases (Brown, 1992; Wright *et al.*, 1993).

Horse gram (*Dolichos biflorus*) is cultivated extensively in India. It is widely used as animal feed and as minor pulse in human nutrition. Raw seeds of horse gram contain antinutritional factors that are inactivated by heat treatment, leading to improvement in the nutritional quality of the seeds. Knowledge of the structure and function of these antinutritional factors can enable one to devise better methods of processing.

Horse gram proteinase inhibitor consists of multiple components of which the major component (peak C) is the predominant form. A protease inhibitor specific to bovine trypsin and chymotrypsin has been purified to homogeneity from horse gram. The protein has a molecular weight of 15 500 and contains two tyrosine residues, two phenylalanine residues, no tryptophan,

and possibly seven disulfide bridges (Ramasarma and Rajagopal Rao, 1991). The horse gram protease inhibitor (HGPI) resembles other Bowman–Birk-type protease inhibitors. These Bowman–Birk-type inhibitors are characterized by low molecular weight, high disulfide content, and low content of aromatic amino acids. The three-dimensional structure by X-ray crystallography of some of the Bowman–Birk-type inhibitors is available, which helps in understanding the structure–function relationship of these inhibitors (Bode and Huber, 1992).

In this investigation, we have looked at the structure and interaction of this major component of HGPI. To understand the structure and interactions of HGPI, the solution conformation of the inhibitor was followed by UV absorption, fluorescence, and circular dichroism measurements. The important dynamic properties such as the interaction of HGPI with trypsin and chymotrypsin have been followed by both kinetics of inhibition and CD measurements and compared with those of other reported Bowman–Birk-type inhibitors.

MATERIALS AND METHODS

Horse gram (*D. biflorus*) was procured from a local market. Trypsin (three times crystallized), chymotrypsin (three times crystallized), DTT, Tris, BAPNA, BTPNA, 2-mercaptoethanol, and standard molecular weight kit dalton marker VI and VII L were obtained from Sigma Chemical Co. (St. Louis, MO). Alkali-soluble casein was from E. Merck (Darmstadt, Germany) and DE 52 from Whatman (Maidstone, U.K.). Sephadex G-50, Pharmacia LMW protein calibration kit, and DEAE-Sephacel were from Pharmacia (Uppsala, Sweden). Guanidine hydrochloride, urea, SDS, and Coomassie Brilliant Blue R-250 were from Pierce Chemical Co. (Rockford, IL). All other chemicals and reagents used are of analytical grade.

HGPI was purified according to the method of Ramasarma and Rajagopal Rao (1991) and used in the present study. The multiple components of HGPI were separated on a DEAE-Sephacel column using ammonium bicarbonate gradient. Polyacrylamide gel electrophoresis with and without SDS was performed according to the method of Laemmli (1970). The inhibitors were visualized on the native gel by the method of

---

* Author to whom correspondence should be addressed (e-mail cftri @ nicfos.ernet.in).

[†] Department of Biochemistry and Nutrition.

[‡] Department of Protein Technology.

Uriel and Berges (1968) with modifications according to Filho and Moreiro (1978).

Trypsin and Chymotrypsin Inhibitor Activities. Caseinolytic activities of trypsin and chymotrypsin and their inhibition by inhibitor was determined according to the method of Kakade et al. (1969). One unit of enzyme activity is defined as an increase in the absorbance of 0.01 at 280 nm under the conditions of assay. One inhibitory unit (IU) is the amount that inhibits the proteolytic activity by one unit. Amidolytic activity of trypsin and its inhibition were routinely assayed using BAPNA as the substrate according to the method of Kakade et al. (1970).

Kinetic Studies. The nature of inhibition of trypsin or chymotrypsin was studied by incubating trypsin or chymotrypsin with various concentrations of BAPNA or BTPNA in the presence and absence of the inhibitor (Kassell, 1970). Competition experiments were done by incubating 50 μg of trypsin or chymotrypsin and a mixture of 50 μg of trypsin and 50 μg of chymotrypsin with various amounts of inhibitor (5–15 μg) separately. Suitable aliquots were withdrawn and assayed for both the inhibitory activities. The number of moles of counterenzyme per mole of inhibitor bound was calculated on the basis of the amount (micromoles) of the counterenzyme inhibited by enzyme inhibitor complex using the above-mentioned inhibitory assay.

Competition experiments were also done by titrating inhibitor with trypsin in the presence and absence of saturating amounts of chymotrypsin and with chymotrypsin in the presence and absence of trypsin.

Absorption spectra were recorded on a Shimadzu UV-160A spectrophotometer. Protein concentrations were calculated using $E^{1\%}_{1cm,280nm}$ as 4.0 for HGPI.

Carbohydrate Content. Carbohydrate was estimated according to the method of Montgomery (1961) using a 1% protein solution.

Phosphorus Content. Phosphorus was estimated according to the method of Taussky and Schorr (1953) using a 1% protein solution in 10 mM Tris-HCl buffer, pH 7.5.

Tyrosine and Tryptophan Content. Tyrosine and tryptophan were estimated according to the method of Edlehoch (1967). Tryptophan content was also estimated according to the method of Spies and Chambers (1949).

Fluorescence Measurements. Fluorescence measurements were made with a Shimadzu RF 5000 automatic recording spectrofluorometer with a 10 nm bandwidth for both excitation and emission monochromators. The emission spectra of the inhibitor at 25 °C in the region 300–360 nm were recorded at pH 7.5 (10 mM Tris-HCl buffer) with protein solutions having an absorbance of less than 0.1 at 280 nm after excitation at 280 nm. The temperature of the cell was maintained by circulating water at that temperature from a constant-temperature circulating water bath.

CD Spectroscopy. Circular dichroism measurements were made with a Jasco J20C automatic recording spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. Dry nitrogen was purged continuously into the instrument before and during experiments. Slits were programmed to yield a 10 Å bandwidth at each wavelength. The measurements were made at 25 °C in 10 mM Tris-HCl buffer, pH 7.5, using a quartz cell of appropriate path length (1 mm path length cell in the far-UV region and 1 cm path length cell in the near-UV region). The mean residue ellipticity values were calculated using a value of 110 for mean residue weight. The secondary structure content was analyzed by the computer program CD PROT (Menendez-Arias et al., 1988). In this method, the secondary structure of protein is calculated as a linear combination of reference spectra based on proteins of known tertiary structure or polyamino acids in different conformations by three methods (Chang et al., 1978; Bolotina et al., 1980; Yang and Kubota, 1985).

Complex Formation. For following the complex formation with trypsin or chymotrypsin, HGPI was titrated with trypsin or chymotrypsin and changes in the ellipticity values of the 280 nm CD band were measured using an inhibitor concentration of 2.1×10^{-5} M and various enzyme concentrations from 0.47×10^{-5} to 3.56×10^{-5} M. Mean residue ellipticity values

(θ_{mrw}) were calculated as described by Jibson et al. (1981). The decrease in the 280 nm CD band with the addition of trypsin or chymotrypsin was analyzed in terms of the binding of the inhibitor to the enzyme using established procedures (Appu Rao, 1992). On the assumption that the binding of each molecule of trypsin or chymotrypsin produces the same degree of reduction in the 280 nm CD band and that the binding is statistical, the intrinsic binding constant K is given by

$$K = [\beta/(1 - \beta)]1/C_f \qquad (1)$$

where β is $[\Delta\theta]/[\Delta\theta]_{max}$, C_f is free enzyme concentration, equal to $(C_T - n\beta T)$, and θ is the ellipticity value at 280 nm at the total concentration of the trypsin C_T. The value T is 2.1×10^{-5} mol for the inhibitor; n is the binding stoichiometry, i.e., moles of trypsin bound per mole of inhibitor.

The best fit to the data was obtained by the method of least squares. To estimate the stoichiometry of binding, the data were analyzed by the equation of Ikeda and Hamaguchi (1969):

$$\log[\Delta\theta/\Delta\theta_{max} - \Delta\theta] = \log K + n \log C_T \qquad (2)$$

RESULTS AND DISCUSSION

The SDS–PAGE pattern of the inhibitor showed a single band having a molecular weight of 15 000, but on native PAGE the inhibitor showed five distinct multiple components. These multiple components were separated by DEAE-Sephacel chromatography (Figure 1). The major component (peak C) was used for all kinetic and spectral studies.

Mode of Inhibition. The double-reciprocal plots of the kinetic data were linear. In the presence of inhibitor, there was no change in the V_{max}, while there is a change in the K_m values. These results are suggestive of competitive type of inhibition for both trypsin and chymotrypsin. The K_i values calculated from these data was found to be 2.75×10^{-7} M for trypsin and 1.0×10^{-7} M for chymotrypsin.

Competition Experiments. The competitive binding measurements were made with the assumption that the inhibitor–enzyme complexes were stable and dissociated very slowly (Bode and Huber, 1992). The purified horse gram inhibitor was found to inhibit both trypsin and chymotrypsin. It was, therefore, of interest to check whether HGPI has the ability to interact simultaneously with both trypsin and chymotrypsin. To investigate this aspect, first HGPI in various concentrations ranging from 0.33 to 1.0 nmol was incubated with trypsin (2.08 nmol) or chymotrypsin (2.2 nmol). From the results obtained in inhibition assays (Table 1), it can be seen that essentially 3 mol of trypsin and 2 mol of chymotrypsin were found to interact with HGPI.

To determine whether all sites are available for binding when both trypsin and chymotrypsin are present together, experiments were carried out in which trypsin and chymotrypsin were added (2 nmol each) with various concentrations of HGPI (0.33–1.0 nmol). The results of the inhibition assay (Table 2) indicated that at all concentrations of the inhibitor approximately 1.6 equiv of trypsin and 1.5 equiv of chymotrypsin were bound to the inhibitor, suggesting that in all only three binding sites are accessible at one time. Therefore, in the inhibitor–enzyme complexes, there may be either two molecules of trypsin and one molecule of chymotrypsin or vice versa.

Further experiments were carried out to determine the accessibility of sites that are present in the inhibitor. In these, at a fixed concentration of the inhibitor, various amounts of trypsin or chymotrypsin were added and the effect of the complex formation on the inhibitory
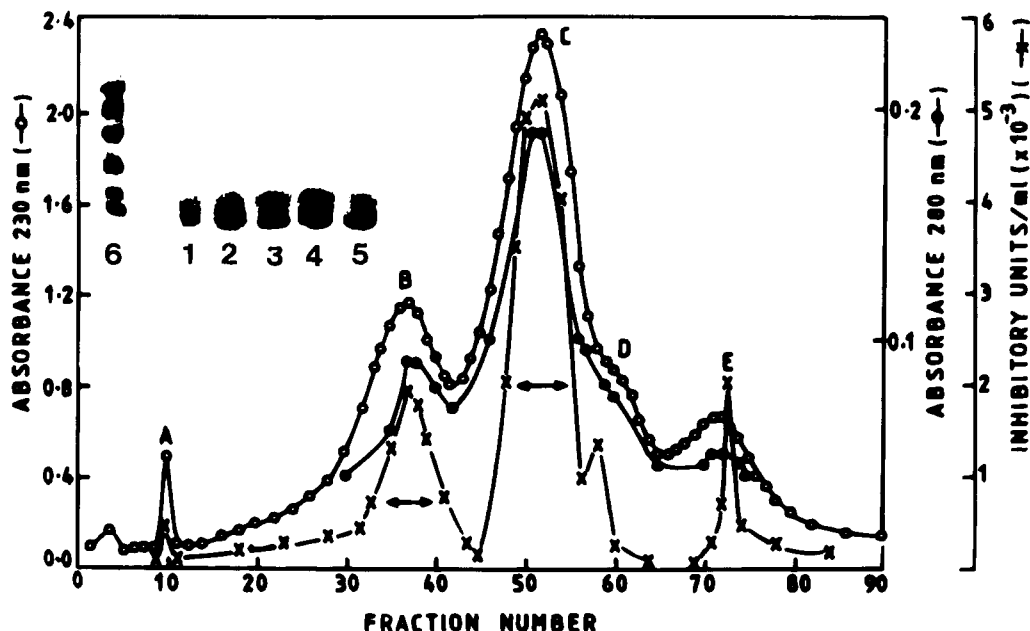
Conformational Studies on HGPI

J. Agric. Food Chem., Vol. 42, No. 10, 1994 **2141**

Figure 1. Separation of isoinhibitors of HGPI by DEAE-Sephacel chromatography: column size, 3.5 × 12 cm; 80 mg of HGPI in 10 mL of 100 mM ammonium bicarbonate buffer; eluent, 0.1–0.4 M ammonium bicarbonate linear gradient (250 mL each); flow rate, 30 mL/h; fraction volume, 5.7 mL. Peak C was pooled, lyophilized, and rechromatographed. (Inset) 8–25% gradient gel pattern (SDS–PAGE), pH 8.1 (Phast system): (lane 6) Pharmacia LMW protein calibration kit; (lane 1) peak E; (lane 2) peak D; (lane 3) peak C; (lane 4) peak B; (lane 5) G-50, purified HGPI.

Table 1. Chymotrypsin Inhibition by Trypsin–HGPI Complex and Trypsin Inhibition by Chymotrypsin–HGPI Complex

HGPI		trypsin bound[b] (nmol)	T/I (mol/mol)	chymotrypsin bound[b] (nmol)	CT/I (mol/mol)
μg	nmol[a]				
5.0	0.333	0.89	2.69	0.61	1.83
7.5	0.500	1.31	2.62	0.90	1.80
10.0	0.666	1.73	2.60	1.22	1.83
15.0	1.000	2.01	2.01	1.60	1.60

[a] Calculations done assuming the molecular mass of HGPI to be 15 000. [b] Average of three determinations. The inhibitor (5.0, 7.5, 10.0, and 15.0 μg) was incubated separately with 50 μg of trypsin (2.08 nmol) and 50 μg of chymotrypsin (2.2 nmol) in 1 mL of 100 nM phosphate buffer, pH 7.6, for 10 min at 37 °C. Immediately after incubation, aliquots were taken and assayed for the residual chymotryptic and tryptic inhibitory activities.

Table 2. Trypsin and Chymotrypsin Inhibition by Trypsin–HGPI–Chymotrypsin Complex

HGPI		trypsin bound[b] (nmol)	T/I (mol/mol)	chymotrypsin bound[b] (nmol)	CT/I (mol/mol)
μg	nmol[a]				
5.0	0.333	0.62	1.86	0.52	1.56
7.5	0.500	0.85	1.70	0.79	1.58
10.0	0.666	1.10	1.65	0.98	1.47
15.0	1.000	1.34	1.34	1.30	1.30

[a] Calculations done assuming the molecular mass of HGPI to be 15 000. [b] Average of three determinations. The inhibitor (5.0, 7.5, 10.0, and 15.0 μg) was incubated with 50 μg of trypsin (2.09 nmol) and 50 μg of chymotrypsin (2.2 nmol) in 1 mL of 100 mM phosphate bufer, pH 7.6, for 10 min at 37 °C. Immediately after the incubation, aliquots were taken and assayed for the residual chymotryptic and tryptic inhibitory activities.

capacity of HGPI on the second protease was tested. The results shown in Figure 2 indicate that at saturating amounts of chymotrypsin there is still 40% tryptic inhibitory activity retained in the inhibitor, whereas at saturating amounts of trypsin only 20% of chymotryptic inhibitory activity remained. From the inhibition experiments described earlier, it was clear that even at

saturating concentration of the inhibitor both trypsin and chymotrypsin activities persisted to the extent of 10–15%. Considering this, one can perhaps conclude that the chymotryptic inhibitory activity remaining in the trypsin–inhibitor complex is only residual or marginal. This would then suggest that in the presence of trypsin all three sites are occupied. It would appear, therefore, that chymotrypsin is unable to displace trypsin or, if it does so at all, it is only very marginally. When chymotrypsin is first presented, it appears to occupy two sites, while the third one is still available for trypsin binding. This perhaps would explain the retention of 40% tryptic inhibitory activity of HGPI–chymotrypsin complex.

Absorption Spectra of HGPI. Absorption spectra of HGPI in the region 340–240 nm are shown in Figure 3A. The protein has an absorption maximum at 274 nm. The ratio of absorbance at 280 nm to that at 260 nm is 1.02, which may be due to the low content of aromatic amino acids or possible contamination with nucleic acids. The absence of carbohydrate and phosphate rules out the possible contamination with nucleic acids in the preparation. The protein has an extinction coefficient of 4.0 ($E_{1cm,280nm}^{1\%} = 4.0$). The protein contains two tyrosine residues, two phenylalanine residues, seven disulfide bridges, and no free sulfhydryl groups per mole of protein. The protein is devoid of tryptophan. Thus, the absorption spectrum of HGPI is dominated by the contribution of tyrosine, phenylalanine, and disulfide linkages.

Spectrophotometric methods of determination of tyrosine and tryptophan indicate that the inhibitor contains two tyrosine residues and no tryptophan. Estimation of tryptophan according to the Spies and Chambers (1949) method confirms that the inhibitor is free from tryptophan residues.

Fluorescence Spectra. The excitation and emission spectra are shown in Figure 3B,C. The protein has an excitation maximum at 280 nm, and the emission maximum is 334–336 nm after excitation at 280 nm.
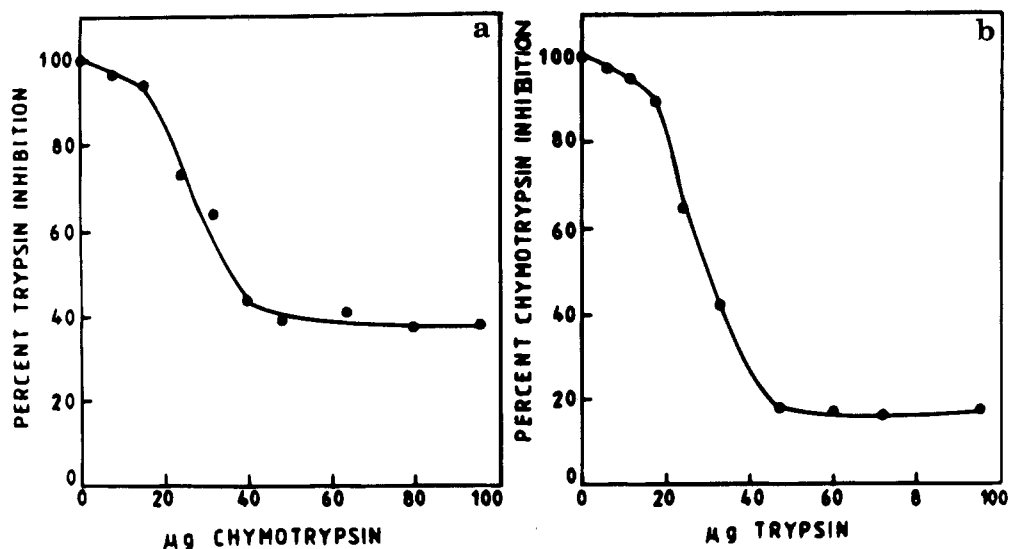
Figure 2. (a) Influence of various amounts of chymotrypsin on the interaction of trypsin with HGPI: 10 μg of inhibitor was titrated with 50 μg of trypsin in the presence of various amounts of chymotrypsin (0−100 μg). (b) Influence of various amounts of trypsin on the interaction of chymotrypsin with HGPI: 10 μg of inhibitor was titrated with 50 μg of chymotrypsin in the presence of various amounts of trypsin (0−100 μg).
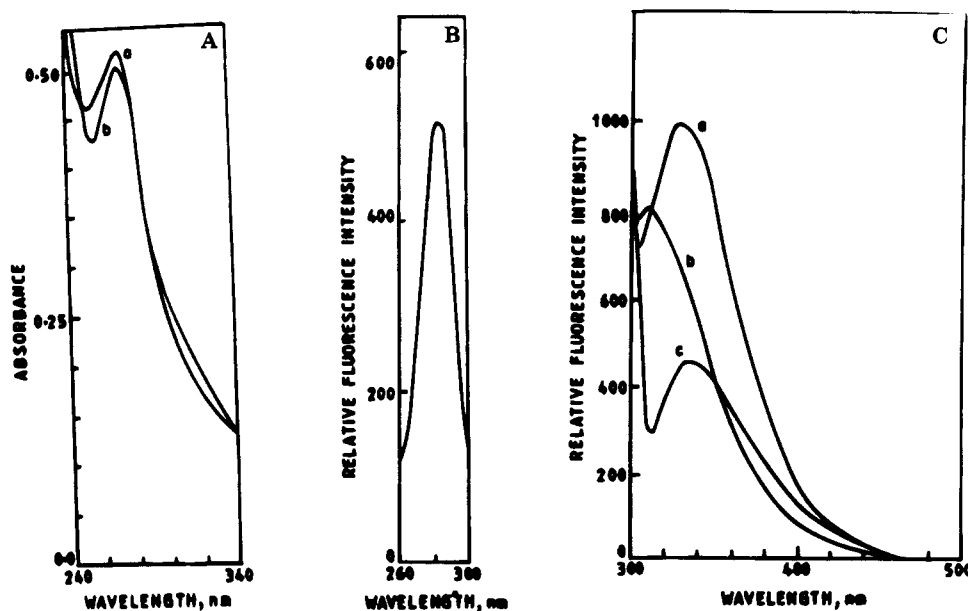

Figure 3. (A) Absorption spectra of HGPI (1 mg/mL solution in 10 mM Tris-HCl buffer, pH 7.5); (B) fluorescence excitation spectra (emission wavelength fixed at 320 nm); (C) fluorescence emission spectra [after excitation at (a) 280, (b) 290, and (c) 295 nm]. Bandwidths for excitation and emission are 10 nm. Spectra were recorded with protein solution having an absorbance of less than 0.1 at 280 nm in 10 mM Tris-HCl buffer, pH 7.5.

The protein emission maximum shifts to 305 nm after excitation at 290 nm. The emission spectrum is essentially due to tyrosine residues since the inhibitor has no tryptophan. The presence of longer wavelength emission for tyrosine suggests the possibility of tyrosinate ion in the molecule. Similar longer wavelength emission for tyrosinate ion is reported for neurotoxins of snake venom (Szabo et al., 1978). The shift of emission maximum toward 305 nm after excitation at 290 nm supports this contention.

CD Spectral Studies of HGPI. The near-ultraviolet circular dichroism spectrum of HGPI at pH 7.5 is shown in Figure 4A. In the region 350−240 nm, the inhibitor exhibits two minima, one at 279 nm and another at 283 nm and a positive band at 249−250 nm. The near-UV CD bands in HGPI are due to tyrosine, phenylalanine, and disulfide linkages (Strickland, 1974). The 279 and 283 nm minima could be due to the two

tyrosine residues in the molecule. The 247 nm band could be due to the contribution of disulfide linkages. The near-UV CD spectrum is very similar to that of Bowman−Birk trypsin inhibitor from soybean (BBTI), which also contains seven disulfide bridges, two tyrosine and two phenylalanine residues, and no tryptophan. HGPI is characterized by a high content of proline and disulfide bonds and a relatively low content of aromatic amino acids. The amino acid composition is very similar to those of soybean and chickpea inhibitors, which makes HGPI structurally analogous to other Bowman−Birk-type inhibitors from the Leguminasae family such as DE3 and DE4 of *Macrotyloma axillare* (Joubert et al., 1979; Townshend et al., 1982; Bewley and Birk, 1978). The negative dichroism above 260 nm in the fragments of BBTI is primarily due to the disulfide bonds with a minor contribution from tyrosine in a hydrophilic environment (Birk et al., 1980). The simi-

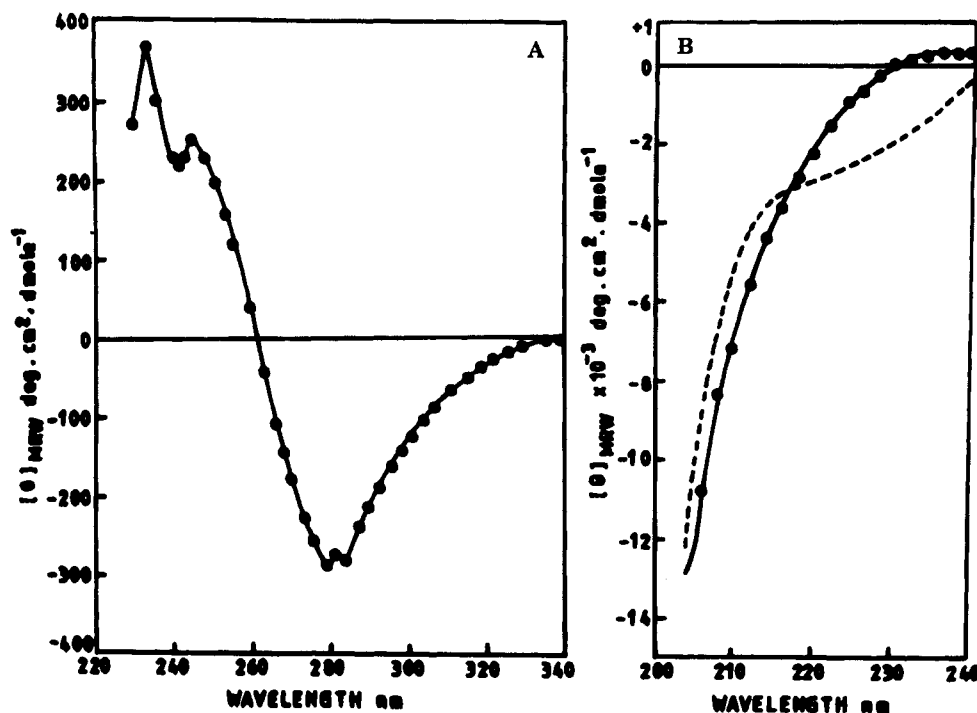Conformational Studies on HGPI

J. Agric. Food Chem., Vol. 42, No. 10, 1994 **2143**

Figure 4. Circular dichroism spectra of HGPI: (A) near-ultraviolet CD spectra (0.6—0.8 mg/mL in 10 mM Tris-HCl buffer, pH 7.5); (B) far-ultraviolet CD spectra (0.1—0.2 mg/mL in 10 mM Tris-HCl buffer, pH 7.5); (●) experimental; (- - -) predicted.

larity of the near-UV CD spectrum to that of a Bowman—Birk-type inhibitor suggests that the tertiary structure of HGPI has a close resemblance to that of BBTI.

The far-UV CD spectrum of the inhibitor in the region 240—200 nm is shown in Figure 4B. The native spectrum has a positive band at 232 nm, a negative shoulder at 223—225 nm, and a broad minima around 205 nm. The CD spectrum is typical of a protein that contains very little ordered structure. The positive band centered around 235—240 nm could be due to the overlapping nature of disulfide bonds and aperiodic structure. The minimum around 205 nm is due to the overwhelming contribution of aperiodic structure.

CD Data Analysis. The secondary structure content of the inhibitor was analyzed by different curve fitting programs. Of the three methods used, the method of Yang and Kubota (1985) gives a good fit to experimental data (Figure 4B). The agreement between the experimental curve and the predicted curve became better for the spectra of HGPI after reduction. This could be due to the contribution of disulfide linkages to the far-UV CD, which is minimal after reduction. According to the analysis based on this method, the protein has 31% β structure and 69% aperiodic structure. Protein does not contain any α-helix. A superficial reading of the spectra indicates the absence of well-defined bands at 222 and 208 nm, lending credence to the analysis that protein is devoid of α-helix. The presence of a trough around 225 nm suggests the possibility of antiparallel β structure in the molecule. The inhibitor also resembles other Bowman—Birk-type inhibitors in terms of secondary structure content as reflected in the far-UV CD spectra. In the case of BBTI, on the basis of 2D NMR analysis (Werner and Wemmer, 1991) it has been concluded that, in addition to two distinct regions of β sheets in each inhibitory domain, two short sheetlike regions have been identified, linking each inhibitory domain in the rest of the molecule. From the NMR measurements, it is not

clear whether the inhibitor forms a continuous four-strand β sheet across both inhibitory domains. The combination of the tight turn and antiparallel β sheet tends to form a β hairpin in each of the inhibitory domains. Thus, it is conceivable that HGPI also contains a good amount of antiparallel β structure as reflected in the analysis. From the CD measurements, Bewley and Birk (1978) have reported that there is no evidence of extensive regions of regular secondary structure in Bowman—Birk inhibitor. The CD spectrum of the HGPI is very similar to CD spectra of trypsin inhibitors from chickpea, adzuki bean, DE3 and DE4 of *M. axillare,* and other Bowman—Birk-type inhibitors (Joubert et al., 1979; Townshend et al., 1982; Bewley and Birk, 1978; Yoshida et al., 1976).

Complex Formation. The complex formation between HGPI and trypsin/chymotrypsin was followed by CD measurements in the near-UV region. The spectra of the inhibitor, trypsin, and HGPI—trypsin complex are shown in Figure 5A. The ellipticity value of the 280 nm band of the inhibitor decreases due to complex formation with trypsin. The spectrum is characterized by an isosbestic point at 263 nm. The rotation at 280 nm decreases with increasing concentration of enzyme. By following the intensity of the CD band as a function of added trypsin concentration, it was possible to calculate the equilibrium constant (K_{eq}) for HGPI. Quantitation of the HGPI—trypsin interaction is shown in Figure 6.

The decrease in the CD amplitude of the 280 nm band increased with trypsin concentration (Figure 6A). The plot of $1/[\Delta\theta]$ vs the reciprocal of trypsin concentration was linear. From the intercept, $[\Delta\theta]_{max}$ at 280 nm was calculated to be 226 deg·cm²·dmol^{-1} (Figure 6B).

The plot of $\log(\Delta\theta/\Delta\theta_{max} - \Delta\theta)$ against $\log C_T$ was a straight line (Figure 6C). The slope was calculated by the method of least squares and found to be 1.05 ± 0.15, which suggests that one molecule of inhibitor was bound to one molecule of trypsin.
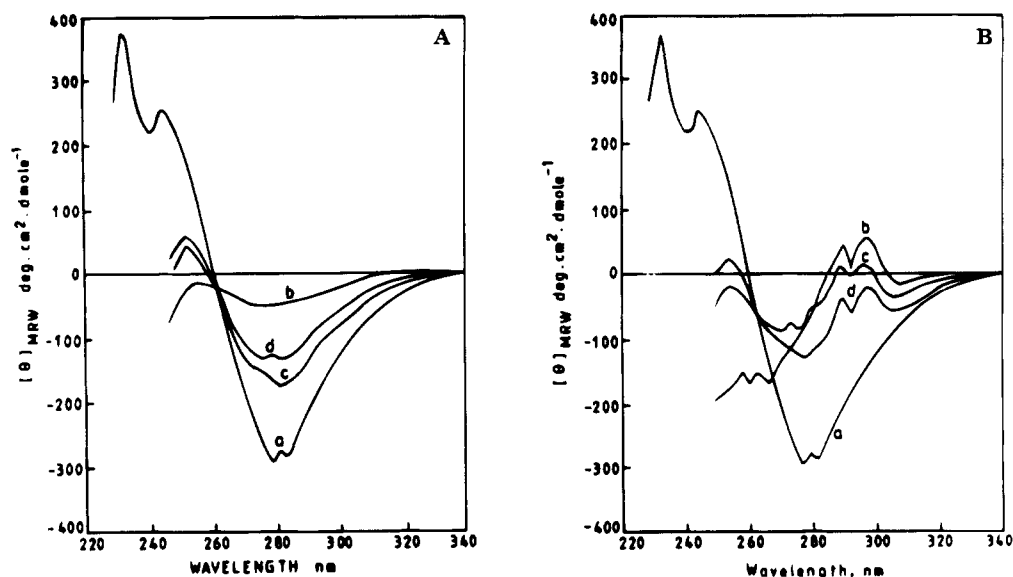
Figure 5. (A) Circular dichroism spectra of trypsin—HGPI complex: (a) native HGPI; (b) trypsin; (c) 0.54 mg of trypsin + 0.35 mg of HGPI/mL; (d) 0.9 mg of trypsin + 0.35 mg of HGPI/mL. (B) Circular dichroism spectra of chymotrypsin—HGPI complex: (a) native HGPI; (b) chymotrypsin; (c) 0.36 mg of chymotrypsin + 0.35 mg of HGPI/mL; (d) 0.6 mg of chymotrypsin + 0.35 mg of HGPI/mL.
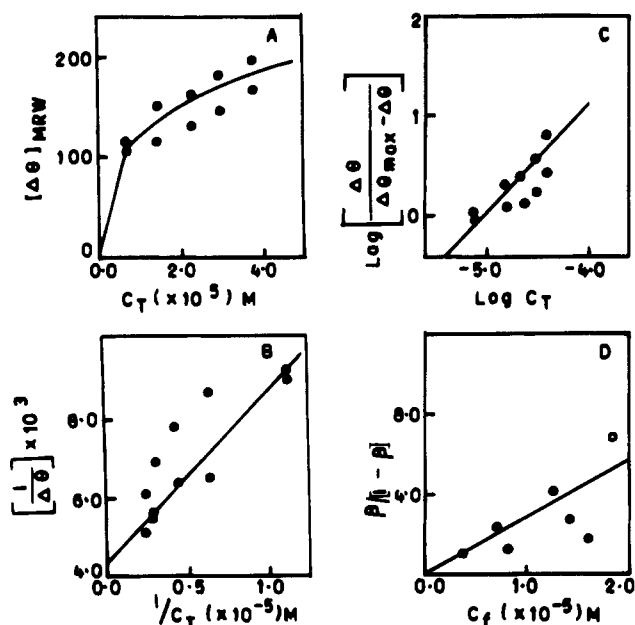

Figure 6. Quantitation of complex formation of HGPI with trypsin: (A) variation of ellipticity values at 280 nm (CD band in the presence of various concentrations of trypsin); (B) double-reciprocal plot of the data; (C) $\log(\Delta\theta/\Delta\theta_{max} - \Delta\theta)$ vs $\log C_T$; (D) $\beta/(1 - \beta)$ vs C_f. Straight lines drawn through the experimental data points are from least-squares analysis.

Figure 7. Quantitation of complex formation of HGPI with chymotrypsin: (A) variation of ellipticity values at 280 nm (CD band in the presence of various concentrations of chymotrypsin); (B) double-reciprocal plot of the data; (C) $\log(\Delta\theta/\Delta\theta_{max} - \Delta\theta)$ vs $\log C_T$; (D) $\beta/(1 - \beta)$ vs C_f. Straight lines drawn through the experimental data points are from least-squares analysis.

Using the value of $[\Delta\theta]_{max}$ at 280 nm as 226 deg·cm²·dmol⁻¹, the equilibrium constant was calculated with eq 1 of Lee *et al.* (1975).

The plot of $\beta/(1 - \beta)$ vs C_f was a straight line (Figure 6D). The K value thus obtained is $(2.9 \pm 0.4) \times 10^5$ mol⁻¹.

The quantitation of HGPI interaction with chymotrypsin is shown in Figure 7. A similar analysis of data gives an association constant of $(5.2 \pm 0.7) \times 10^5$ mol⁻¹.

From the above data, it is clear that HGPI forms complexes with both trypsin and chymotrypsin, with greater affinity for chymotrypsin.

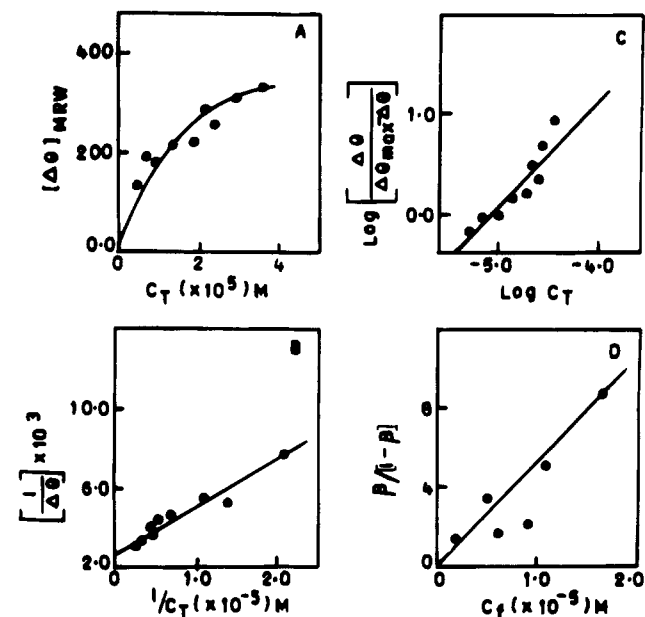The free energy changes calculated with the equation $\Delta G = -RT \ln K$ for the HGPI—trypsin and HGPI—

chymotrypsin interactions are -7.42 and -7.77 kcal·mol⁻¹, respectively. Thus, HGPI has comparable affinity for both trypsin and chymotrypsin. The equilibrium constant and free energy change obtained for HGPI—trypsin/chymotrypsin interaction are comparable to the equilibrium constant and free energy change for Bowman—Birk inhibitor—trypsin/chymotrypsin interaction (Turner *et al.*, 1975).

Both the inhibition kinetic data and the competition experiments indicate a stoichiometry of three for trypsin and two for chymotrypsin. However, in circular dichroism measurements, the molar ratio of the inhibitor to trypsin or chymotrypsin is only 1.2—1.3 and could not be increased due to measurement constraints. Up to

Conformational Studies on HGPI

J. Agric. Food Chem., Vol. 42, No. 10, 1994 **2145**

the concentration we have used, the binding stoichiometry remains the same. It is pertinent to mention that the equilibrium constant is not sensitive to the value of n.

The effect of chymotrypsin and trypsin interaction with HGPI was also followed by CD measurements in the far UV. Analysis of the CD data did not suggest any significant change in the secondary structure of either trypsin or chymotrypsin due to complex formation. Thus, the interaction of HGPI with trypsin and chymotrypsin alters the microenvironment of the inhibitor as reflected in the changes in the ellipticity values of the 280 nm UV CD band.

In conclusion, the near-UV CD bands of HGPI are dominated by tyrosine and disulfide linkages. The spectrum is similar to that of other Bowman–Birk-type inhibitors. Far-UV CD measurements indicated the absence of α-helix and the presence of 30% β sheet, with the rest aperiodic structure. The tyrosine residues are in a hydrophilic environment. The interaction of HGPI with trypsin and chymotrypsin results in the alteration of microenvironment of HGPI without significant changes in the secondary structure of the molecule. The equilibrium constants and free energy changes for the HGPI–trypsin and HGPI–chymotrypsin interactions are comparable to those of other Bowman–Birk inhibitor–trypsin/chymotrypsin interactions and suggest medium affinity toward chymotrypsin and trypsin. Preliminary crystallographic studies have recently been carried out for HGPI by our group (Prakash *et al.*, 1994) and indicated that the crystals belong to cubic space group $P2_13$ ($a = 110.81$ Å)k and diffract X-rays to beyond 3.0 Å resolution. It is likely that the three-dimensional structure of this inhibitor will be available soon for a detailed understanding of the structure and function of the inhibitor and its interaction with trypsin or chymotrypsin.

ABBREVIATIONS USED

HGPI, horse gram protease inhibitor; CD, circular dichroism; SDS, sodium dodecyl sulfate; BAPNA, *N*-benzoylarginine *p*-nitroanilide; BTPNA, *N*-benzoyltyrosine *p*-nitroanilide; DTT, dithiothreitol; $E^{1\%}_{1cm,280nm}$, extinction coefficient of 10 mg/mL protein solution at 280 nm at 1 cm path length; BBTI, Bowman–Birk trypsin inhibitor from soybean; K_{eq}, equilibrium constant.

ACKNOWLEDGMENT

LITERATURE CITED

Appu Rao, A. G. A stoichiometric analysis of bovine serum albumin-gossypol interactions: A fluorescence quenching study. *Indian J. Biochem. Biophys.* **1992**, *29*, 179–182.

Bewley, T. A.; Birk, Y. Circular dichroism spectra of isolated soybean and chick peak trypsin/chymotrypsin inhibitors. *Int. J. Pept. Protein Res.* **1978**, *12*, 249–257.

Birk, Y.; Jibson, M. D.; Bewley, T. A. Circular dichroism spectra of cleavage fragments of soybean trypsin chymotrypsin inhibitor. *Int. J. Pept. Protein Res.* **1980**, *15*, 193–199.

Bode, W.; Huber, R. Natural protein proteinase inhibitors and their interactions with proteinases. *Eur. J. Biochem.* **1992**, *204*, 433–451.

Bolotina, I. A.; Cheknov, V. O.; Lugauskas, V.; Finkel Shtein, A. V.; Ptitsyn, O. B. Determination of protein secondary structure from circular dichroism spectra. *Mol. Biol. (Moscow)* **1980**, *14*, 891–909.

Brown, P. AIDS—The Challenge of the future. *New Sci.* **1992**, *54*, 1–4.

Chang, C. T.; Wu, C. Sc.; Yang, J. T. Circular dichroic analysis of protein conformation: Inclusion of the beta-turns. *Anal. Biochem.* **1978**, *91*, 13–31.

Edlehoch, H. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* **1967**, *6*, 1948–1954.

Filho, J. X.; Moreiro, R. A. Visualization of proteinase inhibitors in SDS-Polyacrylamide gels. *Anal. Biochem.* **1978**, *84*, 296–303.

Ikeda, K.; Hamaguchi, K. The binding of N-Acetylglucosamine to Lysozyme. *J. Biochem. (Tokyo)* **1969**, *66*, 513–520.

Jibson, M. D.; Birk, Y.; Bewley, T. A. Circular dichroism spectra of trypsin and chymotrypsin complexes with Bowman–Birk or chick pea trypsin inhibitor. *Int. J. Pept. Protein Res.* **1981**, *18*, 26–32.

Joubert, F.; Kruger, H.; Townshend, G. S.; Botes, D. P. Purification, some properties and the complete primary structure of two protease inhibitors (DE-3 and DE-4) from *Macrotyloma axillare* seed. *Eur. J. Biochem.* **1979**, *97*, 85–91.

Kakade, M. L.; Simons, N.; Liener, I. E. An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soyabean samples. *Cereal Chem.* **1969**, *46*, 518–526.

Kakade, M. L.; Simons, N.; Liener, I. E. Note on the determination of chymotrypsin and chymotrypsin inhibitor activity using casein. *Anal. Biochem.* **1970**, *33*, 255–288.

Kassell, B. Naturally occurring inhibitors of proteolytic enzymes. *Methods Enzymol.* **1970**, *19*, 839–840.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*.

Lee, J. C.; Harrison, D.; Timasheff, S. N. Interaction of vinblastin with calf brain microtubule protein. *J. Biol. Chem.* **1975**, *240*, 9276–9282.

Menendez-Arias, L.; Gomez-Gotierrez, J.; Garcia-Ferrandez, M.; Garcia-Tejedor, A.; Moran, F. A basic microcomputer programme to calculate the secondary structure of proteins from their circular dichroism spectrum. *Comput. Appl. Biosci.* **1988**, *4*, 479–482.

Montgomery, R. Further studies of the phenol-sulfuric acid reagents for carbohydrates. *Biochim. Biophys. Acta* **1961**, *48*, 591–593.

Prakash, B.; Murthy, M. R. N.; Sreerama, Y. N.; Ramasarma, P. R.; Rajagopal Rao, D. Crystallization and preliminary X-ray diffraction studies on a trypsin/chymotrypsin double headed inhibitor from Horsegram. *J. Mol. Biol.* **1994**, *235*, 364–366.

Ramasarma, P. R.; Rajagopal Rao, D. Nature of the tryptic/chymotryptic inhibitor from Horsegram (*Dolichos biflorus*). *Ind. J. Biochem. Biophys.* **1991**, *28*, 418–424.

Ryan, C. A. Proteinase inhibitor gene families: Strategies for transformation to improve plant defenses against herbivorus. *Bioessays* **1989**, *10*, 20–24.

Ryan, C. A. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol.* **1990**, *28*, 425–449.

Spies, J. R.; Chambers, D. C. Chemical determination of tryptophan in proteins. *Anal. Chem.* **1949**, *21*, 1249–1266.

Strickland, E. H. Aromatic contributions to circular dichroism spectra of proteins. *Crit. Rev. Biochem.* **1974**, *2*, 113–175.

2146 *J. Agric. Food Chem.,* Vol. 42, No. 10, 1994

Ramasarma et al.

Szabo, A. J.; Lynn, K. R.; Krajcarski, D. T.; Rayner, D. M. Tyrosinate fluorescence maximum at 345 nm in proteins lacking tryptophan at pH 7.0. *FEBS Lett.* **1978**, *94*, 249–252.

Taussky, H. H.; Schorr, E. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* **1953**, *202*, 675–685.

Townshend, G. S.; Botes, D. P.; Visser, L. Properties of fragments prepared from Bowman–Birk Inhibitors. *Biochim. Biophys. Acta* **1982**, *701*, 346–356.

Troll, W.; Kennedy, A. R. Protease inhibitors as Cancer Chemopreventive agents. *Cancer Res.* **1989**, *49*, 499–503.

Turner, R.; Liener, I. E.; Lovrien, R. E. Equilibria of Bowman–Birk Inhibitor Association with trypsin and α-chymotrypsin. *Biochemistry* **1975**, *14*, 275–282.

Uriel, J.; Berges, J. Characterization of natural inhibitors of trypsin and chymotrypsin by electrophoresis in acrylamide-agarose gels. *Nature* **1968**, *218*, 578–580.

Werner, M. H.; Wemmer, D. E. ¹H Assignments and Secondary structure determination of the soybean trypsin/chymo-trypsin Bowman–Birk inhibitor. *Biochemistry* **1991**, *30*, 3356–3364.

Wright, C. I.; Geula, C.; Marsel Mesulam, M. Protease inhibitors and indoleamines selectively inhibit choline esterases in the histopathologic structures of Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 683–686.

Yang, J. T.; Kubota, S. In *Microdomains in polymer solutions*; Dublin, P. L., Ed.; Plenum Press: New York, 1985.

Yoshida, C.; Yoshikawa, M.; Takagi, T. Near-UV Circular dichroism of trypsin inhibitor of adzuki beans attributable to disulfide groups. *J. Biochem.* (*Tokyo*) **1975**, *80*, 449–454.